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Structural Information on a Membrane Transport Protein from Nuclear Magnetic Resonance Spectroscopy Using Sequence-Selective Nitroxide Labeling†

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ABSTRACT: The lactose transport protein (LacS) from *Streptococcus thermophilus* bearing a single cysteine mutation, K373C, within the putative interhelix loop 10−11 has been overexpressed in native membranes. Cross-polarization magic-angle spinning nuclear magnetic resonance spectroscopy (NMR) could selectively distinguish binding of ^{13}C-labeled substrate to just 50−60 nmol of LacS(K373C) in the native fluid membranes. Nitroxide electron spin-label at the K373C location was essentially immobile on the time scale of both conventional electron spin resonance spectroscopy (ESR) ($<10^{-8}$s) and saturation-transfer ESR ($<10^{-3}$s), under the same conditions as used in the NMR studies. The presence of the nitroxide spin-label effectively obscured the high-resolution NMR signal from bound substrate, even though ^{13}C-labeled substrate was shown to be within the binding center of the protein. The interhelix loop 10−11 is concluded to be in reasonably close proximity to the substrate binding site(s) of LacS (<15 Å), and the loop region is expected to penetrate between the transmembrane segments of the protein that are involved in the translocation process.

The lactose transport protein (LacS) of *Streptococcus thermophilus* belongs to the class of secondary transport systems comprising several dozen families to date (*1*, *2*). LacS protein is a member of the galactoside−pentoside−hexuronide (GPH) family, which also includes the melibiose carrier protein (MelB) of *Escherichia coli* (*3*). In common with many transport proteins, LacS is predicted to fold into 12 transmembrane helices, as illustrated in Figure 1. Members of the GPH family are characterized by the sequence conservation in the putative α-helices II and XI and in interhelix loop 10−11. Transmembrane segment XI flanking this loop shows, along with putative α-helices II and IV, a strong periodicity in terms of lipid-/non-lipid-facing residues (*4*). This periodicity suggests that the non-lipid-facing side of the helix forms part of the core of the protein that interacts with other hydrophilic regions and/or the carrier ligands. Interestingly, most of the sugar specificity mutants of MelB that have an altered sugar *and* cation recognition map in interhelix loop 10−11 and the transmembrane segment XI (*5*). Although none of the selected sugar specificity mutants has been studied in great detail, the observation that so many of these cluster together in regions that are among the most conserved in the GPH family suggests that they are in or near the substrate binding pocket of the protein (*5*). Consistent with this notion is the observation that mutants carrying substitutions for His-376 or Glu-379 in interhelix loop 10−11 of LacS are highly defective in catalyzing coupled transport (*6*, *7*). Among the mutants in this region that are not defective in transport are K373C (interhelix loop 10−11) and W399C (transmembrane helix XI). Both these mutants can be labeled with fluorophores such as anilinonaphthalenesulfonate maleimide, which can be used to probe for ligand binding (*8*).

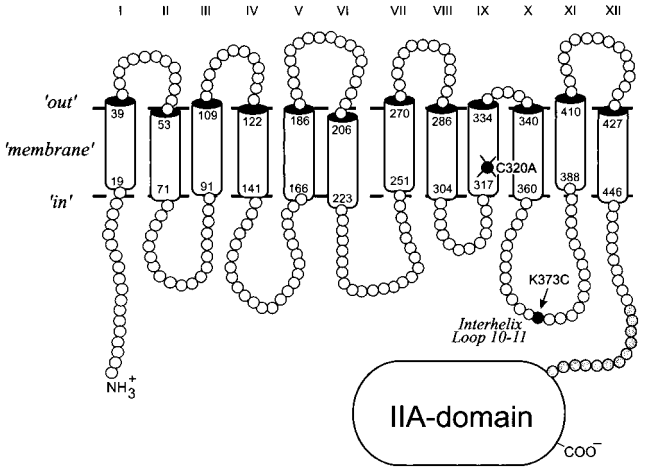

FIGURE 1: Fold of LacS protein predicted from hydropathy analysis, showing the substitution of the native Cys-320 for Ala (C320A) and the location of the single cysteine mutation in K373C. Also shown is the regulatory IIA domain.

Although the analysis of the alkaline phosphatase fusions of MelB suggest that interhelix 10−11 is on the inner surface of the membrane (*9*, *10*), the properties of the LacS position 376 and 379 mutants are more consistent with a location in the interior of the protein (*6*, *7*). Importantly, this region bears some resemblance to a stretch of about 20 amino acids

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present in the LacY protein of *E. coli* that encompasses the sequence motif Lys-X-X-His-X-X-Glu, although the LacY protein is otherwise not homologous to members of the GPH family. Of these residues, the His and Glu residues participate directly in the coupled transport of lactose and have similar roles in both LacY of *E. coli* and LacS in *S. thermophilus*; the Lys residue is not critical and corresponds to residue 373 in LacS (*4*). In contrast to LacS, the proposed location of the equivalent residues in LacY is in the middle of putative helix X (*11*).

Previously, we have demonstrated that solid-state NMR techniques can be used to selectively observe substrate that is immobilized within the binding sites of sugar transport proteins (*12, 13*) or ligands in receptor proteins (*14, 15*), while the proteins remain in a functional state within native fluid membranes. Discrimination of bound ligand or substrate by this approach is based on its dynamic properties alone and so this method is particularly useful for characterizing weak binding processes that are intractable to other means of physical analyses (*13*). However, the main incentive with this approach lies in the possibility of combining labeled ligands with spectroscopic labels in the protein for the purpose of extending the analysis to describe details of the binding center itself. The choice of labels for this strategy is not restricted to nuclear spins, but at the outset, there are some advantages in using species which can exert stronger magnetic interactions. For instance, nitroxide electron spin-labels can be used selectively at specific sites of single cysteine mutations in membrane proteins (*16*). For NMR, the strong dipolar interaction from the electron spin can also circumvent the need for any detailed structural information to guide the selection of sites for labeling.

Here we use MSL[1] attached to the single cysteine mutation in K373C LacS to determine the location of interhelix loop 10−11 with respect to the substrate binding center in the protein. From NMR observations, this loop region is judged to be in reasonably close proximity to substrate that is selectively bound and is expected to be incorporated within the membrane-spanning region of the protein.

MATERIALS AND METHODS

[1-^{13}C]-D-Galactose and nonlabeled sugars were obtained from Sigma (St Louis, MO). The MSL used was 3-maleimidoproxyl, also purchased from Sigma.

Membranes and Sample Preparation. All the procedures to isolate the mutants, to grow *S. thermophilus* ST11(Δ*lacS*) carrying the plasmid pGKhis(K373C), and to isolate right-side-out membrane vesicles have been described (*1, 8*). To remove peripheral membrane proteins as well as cytosolic contaminants, the membrane vesicles were extracted with 5 M urea and 6% (w/v) cholate as described (*17*). The membrane preparations were extensively washed, resuspended in 50 mM potassium phosphate, pH 7.0, and stored in liquid nitrogen following rapid freezing. LacS concentrations used in the studies were determined from the total amounts of protein measured in the membrane preparations (*18*) and from the nominal levels of overexpressed LacS, which were found to be close to 25%, on the basis of densitometric measurements of the membrane proteins fractionated by SDS−PAGE. Thawed membranes were normally equilibrated with 20 mM [1-^{13}C]-D-galactose in the phosphate buffer for at least 30 min at 4 °C with or without the same concentration of nonlabeled sugars for competitive binding studies. Subsequently, the membranes were sedimented by ultracentrifugation (300000*g* for 1 h). The centrifuged LacS membranes were not highly compressed and only 50−60 nmol of LacS could be packed in the NMR sample rotors (0.1−0.2 mL). Occasionally, small volumes (5−10 μL) of substrate solution in the phosphate buffer were mixed directly into the membrane pellet and then allowed to equilibrate at 4 °C.

The LacS mutant preparations were nitroxide-labeled in the presence of 20 mM [1-^{13}C]-D-galactose by incubating the membranes (12 h; 4 °C) with MSL (0.2 mM). Next, the membranes were rinsed extensively by repeatedly resuspending in fresh phosphate buffer containing 20 mM [1-^{13}C]-D-galactose, followed by ultracentrifugation. Equilibrium binding was analyzed from sequential measurements on a sample of LacS membranes equilibrated with [1-^{13}C]-D-galactose at concentrations increasing from 2 to 20 mM. MSL-LacS membranes were chemically reduced by incubating deoxygenated suspensions of the membranes with 100 mM sodium ascorbate (overnight; 4 °C) under nitrogen. Following reductive treatment, membranes were maintained under an atmosphere of nitrogen during all subsequent manipulations and analyses.

Spectroscopy. MAS[1] NMR was conducted on a Bruker MSL spectrometer at 100.6 MHz for carbon-13 (400 MHz for protons), using double-bearing MAS probe heads for 7 mm diameter sample rotors. Sample rotation speeds of 3.0 kHz were used throughout the analysis, while the sample temperature was controlled with the probe bearing gas maintained at 2 °C. CP[1] from proton to carbon spins in the membranes was used to discriminate the portion of substrate immobilized by binding to the membranes. A proton field strength of 50 kHz was used to set up the Hartmann−Hahn match with carbon spins, with a 1 ms contact. The proton field strength was reduced to 25 kHz for decoupling during subsequent signal acquisition. Direct observation of carbon spins was achieved with the same carbon field strengths and decoupling levels as used for CP.

Conventional and ST-ESR measurements were carried out on a Bruker ESR 300 spectometer, operating at 9 GHz (X-band). Sedimented membranes were packed into capillary tubes to give line samples, not exceeding 5 mm, and these were maintained at 2 °C within the standard microwave cavity (TE102). The microwave power level at the samples was adjusted to 0.25 G for all ST-ESR measurements after calibrating the transmitter output with a standard sample of peroxyl disulfonate (*19*). The recording of second harmonic, 90° out-of-phase absorption spectra, their interpretation, and the calibration of rotational mobilities with MSL-hemoglobin in glycerol−water mixtures were as originally described for the ST-ESR method (*19*).

RESULTS AND DISCUSSION

Substrate Binding to LacS Protein. The K373C mutant had lactose transport counterflow activities that are comparable to those of the wild-type LacS protein. Moreover,

[1] Abbreviations: MSL, maleimide spin-label; MAS, magic-angle spinning; CP, cross-polarization; ST-ESR, saturation-transfer electron spin resonance spectroscopy.

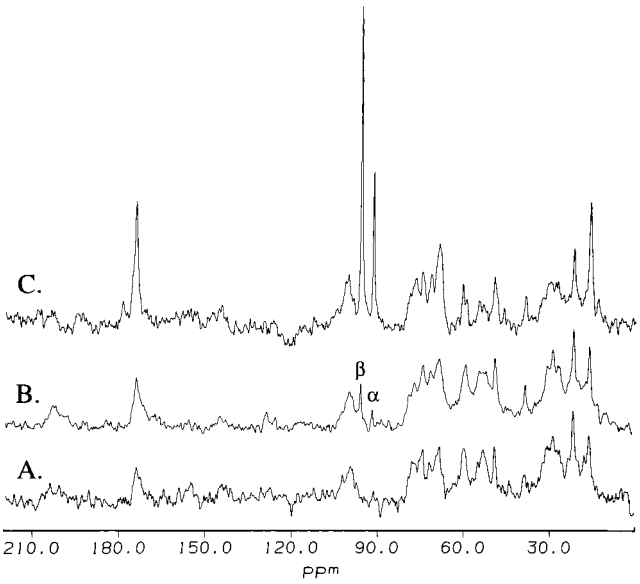

FIGURE 2: CP MAS ^{13}C NMR spectra of membranes at 2 °C containing around 55 nmol of LacS (K373C). (A) Natural-abundance signal from the membranes. (B) Additional signal upon addition of 2 μmol of [1-^{13}C]-D-galactose; the two anomeric states (α and β) are indicated. (C) ^{13}C NMR spectrum recorded from direct (single-pulse) observation, which shows the dominant signal from total labeled sugar added to the system. The CP spectra were recorded from 20 000 acquisitions.

protein activity was not affected by labeling with maleimide labels (data not shown). The CP MAS NMR spectrum of natural-abundance ^{13}C nuclei from LacS membranes contains an unusually high proportion of quite well resolved high-resolution components (Figure 2A). The narrow components below 60 ppm should mostly arise from membrane lipids, while those between 60 and 100 ppm probably originate from the high polysaccharide content of the native host membrane. Contributions from the membrane protein are not expected to be well resolved; these appear as a broad intensity beneath the narrow components between 50 and 70 ppm (mostly from α-carbons) and also from the broadening of the intensity in the alkyl region between 20 and 40 ppm.

On addition of [1-^{13}C]-D-galactose (2 μmol) to the membranes (providing an aqueous concentration in the millimolar range), the CP signal from immobilized sugar appears in the spectral region of 90−100 ppm, showing narrow resonances from both anomers at the labeled C-1 position. Judging by the strong signal observed from total substrate with the nonselective single pulse experiment (Figure 2C), the CP measurement only detects a very small portion of the added substrate. This immobilized population showed no significant difference in chemical shift compared with substrate in free solution (92.4 and 96.5 ppm for the α and β anomers, respectively), which has also been observed for substrate bound to other transport systems (*12*, *13*). The intensity ratio between the anomeric components of bound substrate detected in the CP spectra reflects the approximate 2:1 ratio found in free solution (and also displayed in Figure 2C), and so binding of galactose to the membranes showed no marked preference for either anomer of this substrate.

To investigate the selectivity in the binding measured by these NMR techniques, competitive binding studies were conducted at a 20 mM concentration of each individual sugar. At this concentration, [1-^{13}C]-D-galactose alone provides a

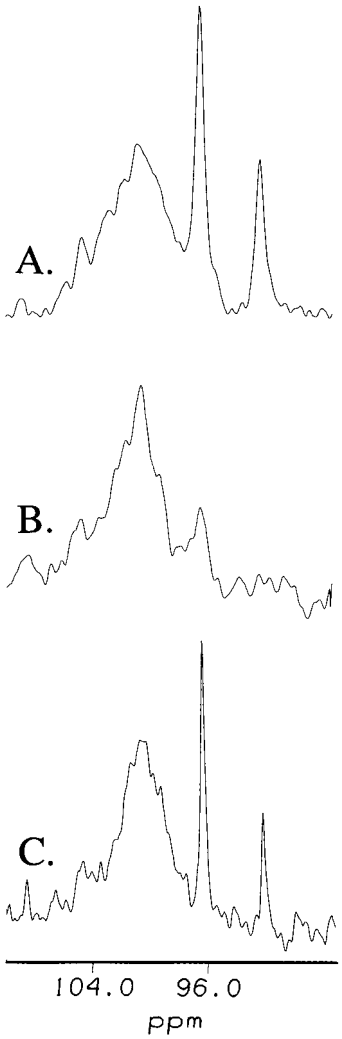

FIGURE 3: Expanded regions of the CP MAS ^{13}C NMR spectra of LacS(K373C)-containing membranes in the presence of 20 mM [1-^{13}C]-D-galactose (A) alone or in combination with 20 mM unlabeled (B) D-lactose or (C) L-galactose. Recording conditions were as described in the legend for Figure 2 or as given under Materials and Methods.

pronounced signal for both anomers, as shown in the expanded region of the CP MAS ^{13}C NMR spectrum in Figure 3A. The signal from the labeled substrate is greatly reduced when combined with an equimolar concentration of the other native substrate, D-lactose, which was not labeled (Figure 3B). However, Figure 3C shows that detection of [1-^{13}C]-D-galactose is largely unaffected by an equimolar concentration of the nontransportable stereoisomer L-galactose, which was also unlabeled. The discrimination between transportable and nontransportable sugars described above shows that the observed binding exhibits a selectivity that corresponds to that for the LacS protein.

In previous studies, we have been able to show that substrate remains associated with other sugar transport systems on a time scale that is long compared with the NMR observation ($>10^{-1}$ s). The persistence of substrate interactions during the NMR measurement is an important prerequisite when attempting to resolve structural features within the protein. The dephasing delayed CP method, introduced to measure rates of substrate exchange (*13*), involves a comparison of spectral recovery rates following destructive dephasing in the NMR experiment. The relatively low

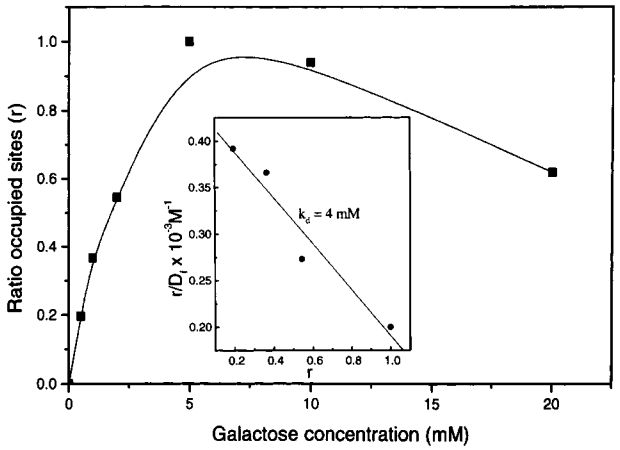

FIGURE 4: Binding isotherm for [1-^{13}C]-D-galactose to LacS membranes as determined from the intensity of the substrate signal in the CP MAS ^{13}C spectra. The ratio of occupied sites used in the binding analysis was simply the intensity of substrate signal expressed as a ratio of the maximum value measured in the titration, which assumes simple binding to a single site in the system. Inset: Scatchard plot of data selected from the NMR analysis of D-galactose binding to LacS membranes.

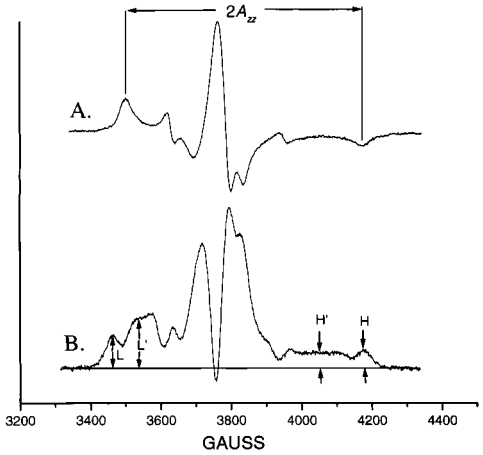

FIGURE 5: (A) Conventional ESR spectrum of MSL-LacS membranes recorded at 2 °C with the maximum splitting being given by the largest component of the nitroxide hyperfine interaction tensor (A_{zz}). (B) ST-ESR spectrum recorded from the MSL-LacS membranes at 2 °C, showing diagnostic peak heights in the low (L′, L) and high (H′, H) field regions, as used in motional analyses (see ref *19*).

sensitivity of the current measurements, with only 50−60 nmol of LacS protein, did not make the reliable quantitation of recoveries from dephased spectra a realistic proposition. Furthermore, other assignable chemical groups, suitable for comparing spectral recovery rates with those of bound substrate, were not readily identifiable in the complex natural-abundance ^{13}C spectra from these membranes. Instead, the characteristics of binding were further explored by titrating the membranes with various concentrations of the labeled substrate. CP is not generally amenable to a quantitative interpretation since its sensitivity is dependent upon the strength of the coupling with other (proton) spins and the various motional processes experienced by these. However, unless exchange or nonspecific binding processes intrude, selectively bound substrate should only experience the invariant dynamic environment of the binding center throughout the titration. In that case, the response within this range of concentrations can be quantitatively compared as illustrated with the following data.

The change in spectral intensity detected from the substrate by CP as it was progressively titrated into the membrane preparation was used to construct the binding isotherm shown in Figure 4. The binding increases to a maximum and then decreases at the higher concentrations used. Importantly, the binding shows saturation kinetics followed by a decrease in binding capacity, presumably due to either some deterioration in the protein activity or an occlusion of binding sites following 30 h of spinning and continuous measurement. Both saturable binding and the moderate loss of binding capacity suggests that the substrate response is confined to specific sites associated with the active transport system. For the purpose of this analysis, the long-term measurements are disregarded and the maximum binding observed is taken to represent a 1:1 binding stoichiometry. The *r* values for the fractional binding are then calculated simply as the fraction of maximum spectral intensity detected from bound substrate. These data were then used to construct the Scatchard plot shown in the inset of Figure 4. The resulting dissociation constant, k_d, of 4 mM falls between the values of 10 and 0.2 mM determined previously for the D-galactose inward and outward transport kinetics, respectively (*7*). The k_d is also comparable to the dissociation constant for thermodynamic binding of D-galactose to the detergent-solubilized LacS protein, estimated to be 2 mM (*8*). Since the binding measured here is saturable and broadly reflects the quantitative binding characteristics described for the LacS protein, it is reasonable to assume that the observations are indeed confined to the binding center in the protein alone and that substrate exchange is again not an important factor in the observations.

Nitroxide Spin-Labeled Membranes. The conventional ESR spectrum of MSL-LacS membranes recorded at 2 °C is shown in Figure 5A and comprises mainly broad components and a full spectral width, arising from the largest component of the nitroxide hyperfine interaction tensor (A_{zz}) of 68 G. This line shape and splitting is characteristic of a nitroxide that is essentially immobile on this time scale ($<10^{-8}$ s) (*19*). The conventional ESR spectrum only shows small contributions from a more mobile spin-label population, appearing as narrower components closer to the central region of the spectrum. The ST-ESR spectrum (Figure 5B) of spin-labeled membranes at 2 °C also provides little evidence of motion, even over the much longer time scale associated with this technique (up to around 10^{-3} s). The large values for the diagnostic peak height ratios shown in both the low-field, L′/L, and high-field, H′/H, regions (see Figure 5B), predict motional rates close to this "rigid limit", when referenced to the *isotropic* calibration obtained from MSL-hemoglobin. The quantitation procedures and calibration data used to interpret rotational mobility were similar to those reported originally for this method (*19*) and are not covered in detail here. The strong immobilization of nitroxide attached to the protein shows that local mobility for this particular MSL is highly restrained and that overall reorientation of LacS in the membranes is restricted, probably due to the membranes being highly rigid at the reduced temperature. These observations show that the dynamics of nitroxide attached to the protein can be disregarded in the interpretation of its paramagnetic effects on NMR observations, as described below.

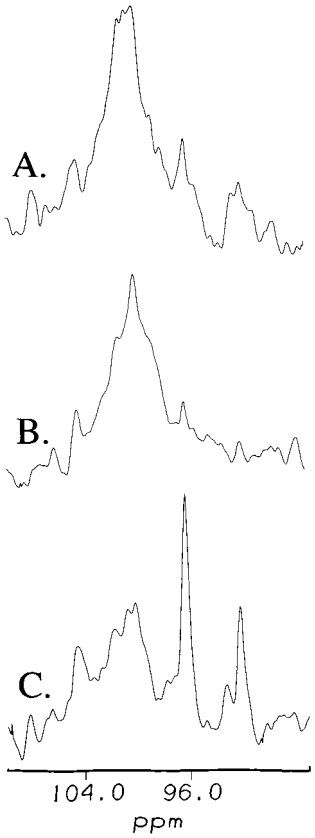

FIGURE 6: Expanded regions of CP MAS ^{13}C NMR spectra recorded from MSL-LacS membranes (A) with 20 mM [1-^{13}C]-D-galactose and (B) after incubation at 60 °C for 5 min, and (C) from MSL-LacS membranes with 20 mM [1-^{13}C]-D-galactose following chemical reduction of MSL (see Materials and Methods).

CP MAS ^{13}C NMR on the membranes with MSL-LacS, equilibrated with 20 mM [1-^{13}C]-D-galactose, showed significant changes in the spectral region displaying the substrate signal (90−100 ppm), when compared with the LacS-containing membranes without MSL. This spectral region, expanded in Figure 6A, shows almost complete suppression of the high-resolution signal from the labeled substrate. Previous ESR observations on this MSL-LacS(K373C), reconstituted into lipid membranes, showed that following brief treatment at 60 °C, the MSL at this interhelix loop became more mobile (Hemminga and Poolman, unpublished results). This treatment did not introduce high mobility in the loop region for MSL-LacS in the *native* membranes according to the ESR observations (not shown), and the substrate signal remained essentially undetectable by NMR as shown in Figure 6B. Chemical reduction of MSL-LacS-(K373C) in the native membranes, however, resulted in complete recovery of the high-resolution signal from the substrate (Figure 6C), demonstrating that substrate is within the binding center of MSL-LacS but that its NMR signal is evidently broadened beyond detection by the paramagnetic interaction with the MSL.

Using the isotropic approximation for the paramagnetic enhancement of transverse relaxation rates, derived from the Solomon equations and applied previously for studies on rhodopsin (*20*), and an estimate of 200 Hz as the upper line width limit for detection of substrate in the NMR measurements, we calculate the MSL to be within about 15 Å of substrate in the binding center of LacS. The long-range dipolar interactions predicted here between spin-label and substrate in this system is due to the absence of rapid reorientational motion within spin-label or the entire protein. Nuclear relaxation effects then rely on the electronic relaxation rate for modulation of the electron−nuclear dipolar interaction, which is on the order of 5×10^{-6} s (*20*). This represents a rather slow modulation of the dipolar interaction within the nuclear environment compared to, for example, those experienced in moderately sized proteins rotating in aqueous solution (*21*) or with paramagnetic species having typically faster rates of electron spin relaxation. The Solomon equations have been shown to provide reasonable predictions for the nuclear relaxation effects from an electron of spin $^1/_2$ in systems that undergo slow molecular motion (*22*, *23*). A more rigorous theoretical treatment of relaxation in "rigid" systems under MAS will be presented by us elsewhere.

Despite the rather large upper limit placed on the electron−nuclear interaction observed here, it may still be assumed that the interhelical loop 10−11 in LacS is folded into the membrane and likely to be located between the transmembrane helices involved in substrate binding. The loop would probably need to penetrate deep into the transmembrane portion of the protein if the attached MSL is required to interfere with the detection of substrate bound to both inner- *and* outer-facing sites, as predicted from the transport kinetics (*7*).

The strength of the paramagnetic interaction in these systems, where a spin-label experiences very low rates of reorientational motion, combined with the relatively slow rates of electronic relaxation for the nitroxide moiety, will not allow quantitation of close interactions (<10 Å) but should permit specific distance measurements to be obtained between more widely spaced segments of the protein sequence, i.e., separated by up to 25−30 Å distance. The large effective range of this interaction removes many of the restraints and requirements for prior knowledge of protein structure, implicit when the weaker internuclear interactions are used, and is expected to prove a valuable approach for determining the overall tertiary structure of proteins in the membrane environment.

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